

Modulatory Effects of Neonatal Exposure to TCDD, or a Mixture of PCBs, *p,p'*-DDT, and *p-p'*-DDE, on Methylnitrosourea-Induced Mammary Tumor Development in the Rat

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The role of organochlorine (OC) exposure in the etiology of breast cancer remains controversial. Thus, our objective was to determine whether the most abundant and toxic OCs found in human milk could, when ingested during the neonatal period, modulate the development of mammary tumors in the rat. We prepared a mixture composed of *p,p'*-dichlorodiphenyltrichloroethane (DDT), its major metabolite, *p,p'*-dichlorodiphenyldichloroethene (DDE), and 19 polychlorinated biphenyls (PCB) based on their concentrations found in the milk of Canadian women. Neonate rats at 1, 5, 10, 15, and 20 days of age were gavaged with this mixture, at 10, 100, and 1,000 times the amount that a human baby would consume. An additional group received 2.5 µg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)/kg body weight (bw) by gavage at 18 days of age, instead of the mixture. On day 21, all treatment groups, except for a control group and a 1,000-mix group, received a single intraperitoneal injection of methylnitrosourea (MNU, 30 mg/kg bw), the initiator of the carcinogenic process. The average number of rats per treatment group was 33. Rats were sacrificed when their tumors reached 1 cm in size, or at 308 days of age. We prepared mammary tumors and mammary gland whole mounts for histologic analysis. There were no significant effects when only the malignant or only the benign tumors were considered. After all benign and malignant lesions were pooled, the number of mammary tumors differed among all MNU-treated groups ($p = 0.02$) with more lesions developing in the MNU-1,000× (median = 4.5; $p = 0.05$) and MNU-TCDD (median = 5.5; $p = 0.07$) compared to the MNU-0 rats (median = 2). Compared to the MNU-0 group, the percentage of rats that developed palpable tumors (benign plus malignant) was slightly higher ($p = 0.06$) in the MNU-TCDD group, but not in the MNU-1,000× group. The percentage of palpable tumors that were malignant was higher ($p = 0.02$) in the MNU-100× group (15/16, 94%) than in the MNU-0 group (10/18, 56%). The highest dose of the mixture delayed ($p = 0.03$) the development of tumors, but this was not observed with the MNU-TCDD treatment. These results suggest that neonatal exposure to high doses of organochlorines could favor the development of MNU-induced mammary lesions, but also delays the development of palpable tumors in the rat. **Key words:** DDE, DDT, mammary tumor, methylnitrosourea, organochlorines, PCB, rat, TCDD. *Environ Health Perspect* 109:739–747 (2001). [Online 13 July 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p739-747desaulniers/abstract.html>

Breast cancer is the most prevalent form of cancer in North American women (1,2). The death rate from breast cancer is decreasing while the incidence is slightly increasing, but this increase is statistically significant only in black American women (2). The acknowledged risk factors for breast cancer are genetic predispositions (3–6) and reproductive factors (7,8). These risk factors account for only 30–50% of the cases (5,9–12); thus, environmental factors (exposure to manmade and natural chemicals, dietary habits, lifestyle) are hypothesized to account for the remaining cases.

The possibility that exposure to environmental contaminants, such as organochlorines, may be linked with breast cancer risk was raised by some (9,13), although others disagree (14–16). Organochlorines include a

large number of agricultural and industrial chemicals and by-products of combustion and incineration. Polychlorinated biphenyls (PCBs), dioxins, and *p,p'*-dichlorodiphenyltrichloroethane (DDT) are lipophilic organochlorines, they accumulate in fat, and they are present at the µg to mg/kg levels in human tissues and milk (17–27), confirming direct exposure of the mammary tissues. Some epidemiologic investigations have found no association, or even a negative one, between human tissue levels of PCB congeners, DDT, and *p,p'*-dichlorodiphenyldichloroethene (DDE) and breast cancer (28–38); but in specific subpopulations (7,22,29,33,34,39–42) or by testing specific congeners (27,33,43,44), some did detect significant associations. The hypothesis of a link between organochlorine exposure and

breast cancer is challenged by the study of accidentally exposed populations. People accidentally exposed to mixtures containing PCBs, polychlorinated dibenzo-dioxins and -furans in central Taiwan in 1979 and in western Japan in 1968 (45,46), and to dioxin in 1976 in Seveso, Italy (47), have not yet been reported to have an increased rate of breast cancer.

Organochlorine compounds have numerous effects *in vivo* and *in vitro*, which suggest that they could modulate the development of mammary tumors. These include antiestrogenic (16) or estrogenic effects (13,48,49) and alteration of thyroid (50–52) and immune functions (53). *In vitro*, these chemicals can decrease (54,55) or increase (13,56) the proliferation of breast cancer cells or immortalized breast epithelial cells, by acting directly on the estrogen receptor or indirectly via metabolic or crosstalk pathways (57,58), or by modulating apoptotic processes (59,60). Deregulation of apoptosis and interference with hormonal actions are key factors influencing mammary tumor development. Animal studies, because of different exposure protocols, demonstrate inconsistencies in linking organochlorine exposure with mammary tumor development. Long-term standard regulatory studies do not support an association between dietary exposure to DDE, DDT, PCBs, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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(TCDD) with endocrine-related cancers in adult animals; however, these studies have limitations (61). DDT can either support the growth of an estrogen-responsive tumor in ovariectomized rats (62) or prevent mammary tumor development in dimethylbenzanthracene (DMBA)-treated rats (63). 3,3',4,4'-tetrachlorobiphenyl (PCB #77) can either potentiate the carcinogenic effect of DMBA (64) or prevent further development of mammary tumors in the rat (65). Acute exposure to TCDD (66) or other aryl-hydrocarbon receptor (Ah-R) agonists (67) in postpubertal rats inhibited mammary tumor development; however, development of the tumors was promoted when TCDD was administered *in utero* (68).

Overall, little attention has been devoted to the effects of perinatal exposure to organochlorines. Excretion of accumulated contaminants through breast milk is a major source of exposure to organochlorines for nursing infants (69). The neonates and fetuses *in utero* are exposed and, in animal studies, accumulate tissue levels higher than those measured in the mothers (70,71). Some have hypothesized that changes in the endocrine environment during the fetal and neonatal periods could increase the probability of future occurrence of breast cancer (22,72–76). We observed that a high dose of a mixture of the most abundant human milk contaminants (19 PCB congeners, *p,p*-DDT, and

p,p-DDE) stimulated the proliferation of breast cancer cells *in vitro* (56). Thus, our objective was to determine whether exposure to this mixture by gavage from neonatal day 1 to day 20, or whether a single exposure to TCDD at neonatal day 18 could modulate the development of methylnitrosourea (MNU)-induced mammary tumors.

Our results suggest that neonatal exposure to this organochlorine mixture (19 PCB congeners, *p,p*-DDT, and *p,p*-DDE) or to a single dose of TCDD (2.5 µg/kg) before MNU treatment favors the development of mammary lesions, but the mixture at a high dose delays the development of palpable tumors in the rat.

Materials and Methods

Animal model. MNU is a DNA-methylating agent believed to induce mammary tumor development by creating *O*⁶-methylguanine in the DNA, which activates the proto-oncogene *Ha-ras* by a G→A transition (second nucleotide) of codon 12 (77–79). It has also been suggested that MNU treatment selects for pre-existing cells with *Ha-ras* oncomutations (80–82). We selected the MNU-treated rat model because, *a*) as in the human disease, MNU-tumors are mostly estrogen dependent; *b*) the carcinomas induced are aggressive and locally invasive; *c*) in contrast to DMBA, MNU has a short half-life (< 30 min) and need not be metabolized to

become active, allowing us to decrease the confounding effects of metabolic enzyme induction by the mixture and by DMBA itself; and *d*) MNU treatment permitted us to study the development of both malignant and benign tumors within a shorter time frame than studies that examined the development of spontaneous mammary tumors, which are mostly benign and take more than 27 weeks to develop (83). Finally, the MNU was injected in prepubertal female rats at 21 days of age, a protocol shown to induce tumors more rapidly than when MNU is injected at 50 days of age (84).

Mixture composition. DDT, DDE, and PCB congeners detected in more than 75% of breast milk samples from Canadian women were included in the mixture according to the median concentrations in milk fat (20,21). Non-*ortho*-chlorinated PCBs (#77, 126, and 169) were also added to the mix, according to the mean (median values were not available) concentrations in human milk fat (19). The doses were prepared to mimic multiples (10×, 100×, and 1,000×) of the typical daily intake of PCBs, DDT, and DDE for a Canadian Caucasian newborn drinking 120 mL breast milk/kg body weight (bw) per day (19). Using the human milk fat concentration of each organochlorine and the milk fat consumption of a newborn [3.7% milk fat (85)], we calculated the daily intake of each organochlorine. All the

Table 1. Concentrations of PCB congeners, DDT, and DDE in the 1,000× dosing mixture.

Family of xenobiotics	Selected congeners ^a	Human milk fat ^b (ng/g)	1,000× Target concentration ^c (µg/mL)	1,000× Analyzed concentration ^d (µg/mL)	1,000× Amount gavaged per dose ^e (µg/kg bw)	
PCBs ^f	Non- <i>ortho</i>	77	0.008	0.03 ^g	0.24	1.22
		126	0.080	0.34	0.32	1.68
		169	0.033	0.14	0.15	0.75
	Mono- <i>ortho</i>	28	8	34.28	33.60	174.05
		66	4	17.14	17.70	91.69
		74	15	64.27	74.30	384.87
		118	16	68.55	70.40	364.67
		156	5	21.42	24.60	127.43
	Di- <i>ortho</i>	99	23	98.54	122.00	631.96
		128	4	17.14	16.10	83.40
		138	27	115.68	124.00	642.32
		153	27	115.68	141.00	730.38
		170	9	38.56	37.60	194.77
		180	18	77.12	81.80	423.72
		183	3	12.85	19.00	98.42
187		7	29.99	34.40	178.19	
194		3	12.85	16.60	85.99	
201		4	17.14	16.10	83.40	
203		3	12.85	13.60	70.45	
DDT		<i>p,p</i> -DDE	233	1,000	1063.00	5506.34
	<i>p,p</i> -DDT	35	149.1	158.00	818.44	
Total xenobiotics		444	1903.67	2064.51	10694.14	

^aIn addition to the non-*ortho* chlorinated PCBs (PCB #77, 126, and 169), only DDE, DDT, and the PCB congeners present in more than 75% of samples from Canadian women were included in the mixture (20,21). Numbering system according to Ballschmiter and Zell (86). ^bMean concentration of non-*ortho* PCBs (19) and median concentration of other PCB and DDT congeners (20,21). ^cDosing mixtures at the 10× and 100× level were prepared from dilution of the 1,000× mixture. The target concentrations are the expected quantities of organochlorines dissolved in corn oil. ^dAnalyzed concentrations indicate the actual congener concentrations in the 1,000× dosing solution, measured in a blind test by Wellington Laboratories (Guelph, Ontario, Canada). ^eAmounts given to the neonates when gavaged with 5.18 mL/kg of the 1,000× mixture at day 1 (a cumulative dose equal to 1,000 times human baby intake at day 0, 1, 2, 3, 4), day 5 (a cumulative dose for day 5, 6, 7, 8, 9), day 10 (a cumulative dose for day 10, 11, 12, 13, 14), day 15 (a cumulative dose for day 15, 16, 17, 18, 19), and day 20 of age (a cumulative dose for day 20, 21, 22, 23, 24). ^fPCB congeners are separated according to the position of the chlorine substitution. ^gThe analyzed concentration is 7.87 times higher than the target concentration. This is the only major deviation from the target concentrations.

chemicals included in the mixture were > 99% pure (AccuStandard Inc., New Haven, CT, USA), and were dissolved in dimethylsulfoxide (DMSO), sometimes following heating at 55°C and sonication for 2 hr, before being diluted in corn oil at 50°C. A blind analysis of the complete mixture was performed to confirm the concentrations of each congener (Wellington Laboratories, Guelph, Ontario, Canada). This analysis revealed a very close match between our target and the analyzed concentrations, except for PCB #77, which had a concentration almost 8 times higher than expected. Some of the discrepancies could be caused by impurities in higher chlorinated PCBs (e.g., PCB #118). The composition and the doses of the high dose mixture are indicated in Table 1.

Animal treatment. Animal treatment was conducted in accordance with the Canadian Council on Animal Care guidelines (86). We could manipulate only a limited number of rats per week, so three litters including only neonate female Sprague-Dawley rats (10–11 females/litter) were prepared every week by the supplier (Charles River, St-Constant, Quebec, Canada), until we accumulated over the weeks approximately 30 female neonates per treatment group. On the day of arrival to the lab, at neonatal day 1 (day 0 is the day of birth), pups from all litters were separated from their dams and regrouped under a heating lamp. They were individually weighed, identified, and exposed by gavage to their first treatment before being returned at random to a fostering dam. Neonates were divided into 7 separate treatment groups: corn oil (0), 1,000×, MNU-0, MNU-10×, MNU-100×, MNU-1,000×, and MNU-TCDD. At ages 1, 5, 10, 15, and 20 days, the neonates received the vehicle or appropriate mixture by gavage, each dose representing 5 days of ingestion (Figure 1). At 18 days of age, the MNU-TCDD group treated only with oil at the above time points, also received 2.5 µg TCDD/kg bw, dissolved in corn oil by gavage [a dose 4 times smaller

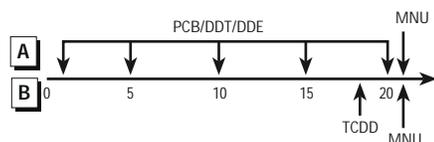


Figure 1. Exposure protocol. (A) Neonates at 1, 5, 10, 15, and 20 days of age were dosed by gavage with the PCB/DDT/DDE mixture at 10, 100, and 1,000 times the amount that a human baby would consume. (B) An additional group received 2.5 µg TCDD/kg bw by gavage at 18 days of age, instead of the mixture. On day 21 of age, all treatment groups, except for a control and a 1,000×-mix group, received an ip injection of MNU (30 mg/kg bw).

than one previously shown to inhibit mammary tumor development in the day 50 DMBA-induced rat model (66). On day 21 of age, all treatment groups, except the 0 and the 1,000× group, received a single intraperitoneal (ip) injection of MNU (30 mg/kg bw, dissolved in 0.9% NaCl, acidified to pH 4.0 with acetic acid), according to previous recommendations (87). The 0 and 1,000× groups received the vehicle only. Rats were weighed before each treatment and then every 10 days. The time of vaginal opening (assessed daily starting at day 25) was recorded as an indicator of puberty. Twice a week, beginning 4 weeks after the MNU injection, rats were manipulated to detect palpable tumors (the date, the location, and size of the tumor were recorded). Seven and nine rats from the 0 and 1,000× groups, respectively, were sacrificed between 55 and 62 days of age, and the remaining rats from these groups were sacrificed at 224 days of age. MNU-treated rats were sacrificed when their palpable tumors reached 1 cm, or by 308 days of age if no palpable tumor was detected. Rats were sacrificed by exsanguination between 0900 and 1200 hr, under isoflurane anesthesia.

Analysis of the mammary gland structures. We recorded the locations and dimensions of all palpable tumors from the time of detection until necropsy. During necropsy, the position and size of the tumors were noted and the tumors were excised from the mammary tissue, weighed, and cut in half, with one-half fixed in 10% neutral buffered formalin (NBF) and the other half frozen in liquid nitrogen. Paraffin sections (5 µm) of the fixed mammary tumors were prepared and stained with hematoxylin and eosin for histologic classification.

We prepared whole mounts by fixing the skin pelts in 10% NBF for 24–48 hr, and then dissected all the mammary tissue. We placed the left thoracic glands in tissue cassettes for later histologic analysis, and de-fatted the rest of the mammary tissue in acetone for 6 days and hydrated it with 100%, 95%, and 70% ethanol for 1 hr each and with water for 30 min. We stained the tissues by immersing them in Alum Carmine Stain (0.4%) containing 0.015% thymol for 2 days. The glands were dehydrated (30 min in water followed by 1 hr each in 70%, 95%, and 100% ethanol) and immersed in xylene for a minimum of 1 hr. We then sealed the stained mammary preparations in plastic pouches containing methyl salicylate.

We observed all abdominal-inguinal and the right cervical-thoracic mammary glands carefully under a stereoscope and then under a microscope at 4× magnification. We noted and identified the various mammary structures according to procedures described by

Russo and Russo (88). Structures that could not be identified by analysis of the whole mounts were dissected and transferred to paraffin blocks before being classified by histology (Table 2). In each animal we counted individually the number of abnormal structures—including intraductal proliferations (iDPs), microtumors, adenomas, hyperplastic alveolar nodules, cysts, and other cystic structures (buds, clumps, nodules, ducts, lobules, milk-filled cysts).

Statistical analysis. We performed all analyses using JMP software (89). To analyze the time of vaginal opening and the body weight (only when specific days were considered), we tested homogeneity of variance by O'Brien and Brown-Forsythe tests, and log-transformed the data when required (body weight on day 40) before conducting analysis of variance (ANOVA). Significant ANOVA were followed by the Tukey-Kramer all-comparison test to identify groups with significantly different means. We analyzed body weight changes throughout the experiment by ANOVA on repeated measures followed by contrast procedures to determine differences between MNU- and non-MNU-treated groups or to assess differences between the MNU-1,000× group and the other MNU groups. We used the nonparametric median test to identify treatment group differences in the number of rats that developed more lesions than the median of all groups (Table 2). We used chi-square analyses to compare the percentage of rats developing tumors among dose groups (Table 3). We assessed differences in the cancer incidence curves by survival curve analysis, using the log-rank test, which places more weight on later delay to tumor, and the Wilcoxon test, which places more weight on early delay to tumor. Significant differences were detected when $p < 0.05$, whereas a $p < 0.1$ suggested a tendency.

Results

Treatment effects on body weight are shown in Figure 2. There were no significant effects of the 1,000× dose compared to the 0 dose on body weight throughout the study. The TCDD treatment decreased growth rate, even before the MNU injection, as indicated by the smaller body weights of these rats on days 20 and 21 ($p = 0.0002$) compared to those of all other groups. These early effects of TCDD on body weight did not persist, so that by day 40 these rats were similar in weight to the other MNU-treated rats. The MNU-treated rats, regardless of the dose of the mixture, had smaller body weights (ANOVA on repeated measures, with contrast, $p = 0.04$) than the 0 and the 1,000× groups throughout the study, and this effect was significant as early as day 30 ($p = 0.0001$). Figure 2

shows that the MNU-1,000× group had the smallest body weights from approximately day 75 until the end of the study, but these were not significantly different from those of the other MNU-treated rats.

The age at the time of vaginal opening was similar in the 0, 1,000×, and the MNU-1,000× dose groups (Figure 3). Except for the MNU-1,000× group, vaginal opening was delayed in all the MNU-treated rats, but significantly ($p = 0.02$) only in the MNU-10× and the MNU-100× groups.

Histologic analysis of all palpable tumors and small mammary lesions dissected from the whole mount preparations revealed the existence of different types of benign and malignant mammary lesions in the rat (Table 2). The groups not treated with MNU (0 and 1,000×) showed very few mammary lesions compared to all the groups treated with MNU. The most abundant lesions were benign hyperplasia of mammary cells within terminal end buds, identified as iDPs, and hyperplasia of alveolar and lobular structures. After all benign and malignant lesions were pooled, the number of rats that developed a number of lesions above the median value significantly differed among MNU-treated groups (Table 2, $p = 0.02$), with more lesions developing in

the MNU-1,000× (median = 4.5; $p = 0.05$) and MNU-TCDD (median = 5.5; $p = 0.07$) than in the MNU-0 rats (median = 2).

Each point on the mammary tumor incidence curves (Figure 4) represents the cumulative percentage of rats that developed palpable tumors (benign and malignant; not including the microscopic lesions analyzed from the whole mount preparations) over time relative to the MNU injection (time = 0). Although the incidence curve of the MNU-TCDD group tended to differ ($p = 0.06$) from that of the MNU-1,000× group, none of the incidence curves differed significantly from that of the MNU-0 group. However, if the data are censored at 150 days [approximately the last day in a similar experiment (66)], the delay in the development of tumors in the MNU-1,000× dose group becomes statistically significant ($p = 0.03$). In addition, Figure 4 demonstrates that at the end of the experiment the percentage of rats that developed palpable mammary tumors was slightly increased ($p = 0.06$) by the MNU-TCDD (18/32 = 56% rats with palpable tumors), but not by the other treatments (MNU-1,000× 13/34, 38%; MNU-100× 11/31, 36%; MNU-10× 10/28, 36%) compared to controls (MNU-0 14/41, 34%).

The percentage of palpable tumors that were malignant was higher ($p = 0.02$) in the MNU-100× group (15/16, 94%) than in the control group (10/18, 56%). In the MNU-10×, MNU-1,000×, and MNU-TCDD groups, these values were 70% (7/10), 73% (11/15), and 72% (18/25), respectively. These palpable tumors, whether malignant or benign, were more often located in the abdominal-inguinal region (66% of the cases) than in the thoracic region ($p = 0.002$).

Table 3 reports the percentage of rats with specific mammary lesions (palpable and dissected from the whole mounts) per treatment. There were no significant effects of any of the organochlorine treatments on the percentage of rats that developed lesions among the MNU-treated groups. However, the MNU treatment increased ($p < 0.05$) the percentage of rats that developed mammary lesions compared to the 0 or 1,000× groups. The presence of cystic structures was common (~ 40%) among the 0 and the 1,000× rats, and this incidence was further increased ($p = 0.03$) by the MNU treatment.

The percentage of rats with nonmammary tumors and/or organ abnormalities was not altered by the 1,000× treatment, but was significantly increased by the MNU treatment (Table 4). Of particular

Table 2. Total number, median, and range of mammary lesions^a per group.

Mammary lesion		MNU-treated mixture							<i>p</i> -Value ^b
		0 (<i>n</i> = 30)	1,000× (<i>n</i> = 33)	0 (<i>n</i> = 41)	10× (<i>n</i> = 28)	100× (<i>n</i> = 31)	1,000× (<i>n</i> = 34)	TCDD (<i>n</i> = 32)	
Benign + malignant	<i>n</i>	1	3	238	206	487	268	524	0.02
	<i>m</i>	0	0	2	2	1	4.5	5.5	
	<i>r</i>	1	2	51	69	170	44	116	
Benign	<i>n</i>	1	0	17	17	6	14	13	0.24
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	1	0	3	2	1	3	2	
Papilloma	<i>n</i>	0	0	7	3	14	1	50	0.32
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	0	0	4	3	11	1	20	
Adenoma	<i>n</i>	0	0	7	13	5	16	14	0.68
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	0	0	2	6	2	7	3	
iDP ^c /hyperplasia ^d	<i>n</i>	0	2	118	144	335	156	331	0.73
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	0	2	28	64	140	37	61	
Total	<i>n</i>	1	2	149	177	360	187	408	0.12
	<i>m</i>	0	0	1	1.5	1	2	3.5	
	<i>r</i>	1	2	34	64	140	37	82	
Malignant	<i>n</i>	0	1	73	12	109	67	85	0.55
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	0	1	50	4	54	43	41	
Adenocarcinoma	<i>n</i>	0	0	16	17	18	14	31	0.64
	<i>m</i>	0	0	0	0	0	0	0	
	<i>r</i>	0	0	4	4	4	2	12	
Total	<i>n</i>	0	1	89	29	127	81	116	0.65
	<i>m</i>	0	0	0	1	0	0	0	
	<i>r</i>	0	1	51	5	54	43	53	

Abbreviations: *m*, median (the median is preferred because it is not drastically affected by extreme values in non-normally distributed population as is the mean); *n*, number of lesions per treatment group; *r*, range of lesions per treatment group (largest value – smallest value).

^aPalpable tumors plus those detected by a microscopic analysis of the whole mounts. ^b*p*-Value of the median test only among the MNU-treated rats. ^cPreneoplastic lesions that, although benign, may grow to produce carcinomas. ^dHyperplasia of alveolar and lobular structures.

importance was the induction of 12 renal tumors that were not related to organochlorine treatment.

Discussion

The results suggest that TCDD and the highest dose of the PCB-DDT-DDE mixture could modulate the development of MNU-induced mammary tumors in the rat. The MNU-TCDD and the MNU-1,000× groups had similar effects in increasing the median number of mammary lesions (Table 2), but the MNU-1,000× group, in contrast to the MNU-TCDD group, transiently delayed the development of palpable tumors.

Transient inhibition in the development of palpable mammary tumors. The transient inhibition in the development of palpable mammary tumors observed in the MNU-1,000× group but not in the MNU-TCDD group (Figure 4) is a phenomenon similar to that observed by Holcomb and Safe (66), but is probably not solely attributable to the activation of the Ah-R. After 10 µg/kg bw TCDD was administered at day 50, a dose 4 times higher than ours, Holcomb and Safe (66) observed at the end of their relatively short experiment (140 days of age) that only 1/10 TCDD-treated rats, compared to 5/10

controls, developed DMBA-induced palpable mammary tumor. Although they concluded that TCDD prevented mammary tumor development in the rat, perhaps they would have observed only a transient delay if they had performed a longer experiment. In our experiment, the MNU-TCDD treatment involved the administration of 87.5 ng TCDD (or 2.5 µg/kg bw) at day 18, and this did not inhibit tumor development. Three times less TCDD-toxic equivalents [TEQ (90, 91)] were administered to the MNU-1,000× group (31.44 ng TCDD-TEQ divided in five doses over the first 20 days of life) than with the MNU-TCDD treatment, yet the MNU-1,000× group showed delayed tumor development. [The TCDD-TEQ value for the mixture was calculated using the mean volume of the mixture administered to the rats, the content of our mixture in Ah-R agonists, and their toxic equivalency factors (TEF) (PCB #77, 0.0001; #126, 0.1; #169, 0.01; #118, 0.0001; #156, 0.0005; #170, 0.0001, #180, 0.00005 (92)]. Therefore, although the dosing regimes differ, mechanisms other than those directly related to the Ah-R binding might be involved.

The development of tumors induced by MNU or DMBA treatments is predominantly

estrogen dependent (93–95). However, the transient inhibition in the detection of palpable tumors in the MNU-1,000× group might not be linked to reduced ovarian estrogen output or to anti-estrogenic effects. Vaginal opening in the rat indicates puberty and estrogen rise during the first estrous cycle. The time to vaginal opening was not delayed in the MNU-1,000× group; in fact, it was accelerated compared to the MNU-10× and MNU-100× groups (Figure 3). Also, ovarian follicular development was not altered by the 1,000× treatment in 21- and 224-day-old rats (96), suggesting that ovarian estrogen output might not be altered. Holcomb and Safe (66) suggested that the antitumorigenic effects of TCDD are attributable to anti-estrogenic effects. TCDD and AhR agonists (65,67) exert antiestrogenic activity not by binding the estrogen receptor, but indirectly by inducing phase I and phase II drug-metabolizing enzymes [reviewed in Schrenk (57)], by down-regulation of the estrogen receptor (ER) and via crosstalk between the AhR and ER signaling pathways (58,97). However, antiestrogenic effects of TCDD depend on dose, tissue, species, and age, and are not detectable in 21-day-old rats (98). At high dose the PCB-DDT-DDE

Table 3. Number of rats with mammary lesions^a per group.

Mammary lesion		0 (n = 30)	1,000× (n = 33)	MNU-treated mixture				TCDD (n = 32)	p-Value ^b
				0 (n = 41)	10× (n = 28)	100× (n = 31)	1,000× (n = 34)		
Benign + malignant									
	<i>n</i>	1	2	28	24	22	25	24	0.54
	%	3.3	6.1	68.3	85.7	71.0	73.5	75.0	
Benign									
Fibroadenoma	<i>n</i>	1	0	12	13	6	9	10	0.25
	%	3.3	0	29.3	46.4	19.4	26.5	31.3	
Papilloma	<i>n</i>	0	0	3	1	3	1	5	0.33
	%	0	0	7.3	3.6	9.7	2.9	15.6	
Adenoma	<i>n</i>	0	0	5	4	4	8	6	0.70
	%	0	0	12.2	14.3	12.9	23.5	18.8	
iDP/hyperplasia	<i>n</i>	0	1	16	9	10	14	15	0.73
	%	0	3.0	39.0	32.1	32.3	41.2	46.9	
Total benign	<i>n</i>	1	1	26	18	17	24	22	0.72
	%	3.3	3.0	63.4	64.3	54.8	70.6	68.8	
Malignant									
Carcinoma <i>in situ</i>	<i>n</i>	0	1	5	5	8	7	4	0.55
	%	0	3.0	12.2	17.9	25.8	20.6	12.5	
Adenocarcinoma	<i>n</i>	0	0	11	12	10	12	13	0.64
	%	0	0	26.8	42.9	32.3	35.3	40.6	
Total malignant	<i>n</i>	0	1	15	15	12	15	14	0.70
	%	0	3.0	36.6	53.6	38.7	44.1	43.8	
Other mammary observations									
Cysts	<i>n</i>	11	13	29	18	19	22	17	0.65
	%	36.7	39.4	70.7	64.3	61.3	64.7	53.1	
Fibrosis	<i>n</i>	1	0	24	21	18	19	18	0.51
	%	3.3	0	58.5	75.0	58.1	55.9	56.3	
Lactational changes ^c	<i>n</i>	2	0	21	17	17	18	11	0.30
	%	6.7	0	51.2	60.7	54.8	52.9	34.4	
Total	<i>n</i>	11	13	32	23	25	28	25	0.98
	%	36.7	39.4	78.1	82.1	80.7	82.4	78.1	

Abbreviations: *n*, number of rats; %, percent of group.

^aPalpable tumors plus those detected by a microscopic analysis of the whole mounts. ^bThe *p*-value corresponds only to analysis of organochlorine effects within the MNU-treated rats; given the large differences between MNU- and non-MNU-treated rats, the comparison of the seven groups was always highly significant (*p* < 0.006), even for the incidence of cysts (*p* = 0.03), which is also elevated in the 0 and 1,000× groups. ^cObservation of milk secretions at necropsy or from the histologic analysis.

mixture stimulated the proliferation of MCF7-E3 cells *in vitro*, but had no uterotrophic effects *in vivo* in immature female rats (56). Thus, effects of the mixture and TCDD on the development of the mammary lesions may not be related only to the estrogenic or antiestrogenic properties of the chemicals.

Body weight differences among groups of rats on the order of 15–20% are known to inhibit mammary tumorigenesis (99). The body weights of rats treated with MNU-1,000× were less than 9% smaller than the control group and were not significantly different from the other MNU-treated rats (Figure 2). We observed no inhibition of tumor development in the MNU-TCDD group even though the body weight was 18% smaller than that of the control group on day 30 [this difference decreased with aging (Figure 2)]. Thus, body weight differences cannot explain the transient inhibition in the detection of palpable tumors.

Increased number of mammary lesions.

In the absence of MNU, the mixture of PCBs, DDT, and DDE does not induce the development of abnormal mammary structures (Table 2 and 3). Similarly, over a longer experiment, Kociba et al. (100) observed no increase in mammary tumors in the rat after dietary administration of 0.1 µg TCDD/kg/day from 7 weeks of age up to 2 years. They even reported a decreased incidence of spontaneous mammary tumors, but at a toxic dose that decreased the fertility of the rat (101) and induced hepatocarcinomas

(100). Thus, the mammary gland does not appear to be a prime target for these chemicals. However, the increased median number of mammary lesions induced by MNU-TCDD and MNU-1,000× (Table 2) and the increased percentage of rats that developed palpable mammary tumors in the MNU-TCDD group (Figure 4) suggest that these treatments favored the initiation and/or promotion phases of the carcinogenic process. Others found that the carcinogenic potential of chemical initiators (DMBA or MNU) is increased by the administration of TCDD *in utero* (68), or at relatively low doses of PCB #77 (64), compared to high [PCB #77 (65); TCDD (66)] or repetitive doses (67) of Ah-R agonists, which reduced tumor development. Brown et al. (68) suggested that TCDD treatment *in utero* potentiates the carcinogenic effects of DMBA by increasing the number of terminal end buds (TEBs) at the time of the DMBA injection at 50 days of age; the TEBs are suggested to be the target sites for the carcinogenic effects of chemical initiators (102). We observed no effects of the mixture on the size of the mammary glands or the number of TEBs at 21 days of age, the time of MNU injection (96). *In vitro*, a malignant transformation bioassay demonstrated that without pretreatment with initiating agents, no malignant foci are observed after continuous TCDD/PCB treatments; but extremely low concentrations of TCDD or PCB #126 (10^{-13} M), after initiation, promote malignant transformation (103). Collectively these results and

ours suggest the existence of multiple time- and dose-dependent mechanisms modulating the development of chemically induced mammary tumors.

Tumor location and phenotype. In our study, most of the palpable tumors were from the abdominal-inguinal glands. In other studies in which tumors were initiated with DMBA (68) or MNU (86,104) at day 50 of age, tumors were located mostly in the thoracic region. Asynchronous postnatal development of the cervical-thoracic versus abdominal-inguinal glands was suggested to explain the predominance of thoracic tumors (88). We are not aware of other articles reporting the location of mammary tumors induced with MNU at 21 days of age in long-term studies. Perhaps at this age, the number of target cells for MNU is larger in the abdominal-inguinal chains of mammary glands than in the thoracic-cervical ones.

The percentage of malignant tumors was significantly higher in the 100× treatment group than in the control group, suggesting that the phenotype of the tumors might be modified by the mixture treatment. Nesaretnam et al. (64) observed that DMBA-induced tumors from rats treated with PCB #77 were mostly of the invasive phenotype. This observation is further supported by the fact that extremely low concentrations of TCDD or PCB #126 (10^{-13} M) promoted malignant transformation (103). In our study, changes in tumor phenotype did not follow a dose relationship, so this observation must be confirmed by other studies.

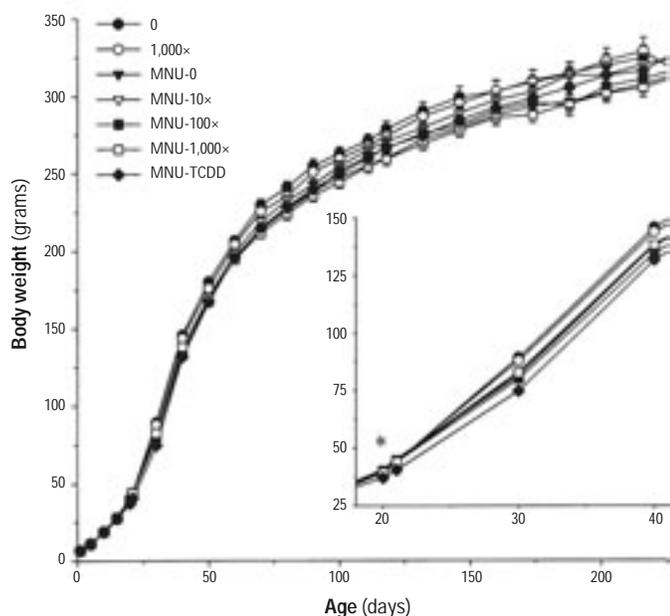


Figure 2. Effects of treatment on body weight. The 0 and 1,000× groups had similar body weights throughout the experiment. All groups treated with MNU had significantly lower body weights than the 0 or 1,000× groups. The inset highlights a significant reduction in body weight starting at day 20 (*) in the MNU-TCDD group, before MNU injection, and at day 30 in all groups treated with MNU.

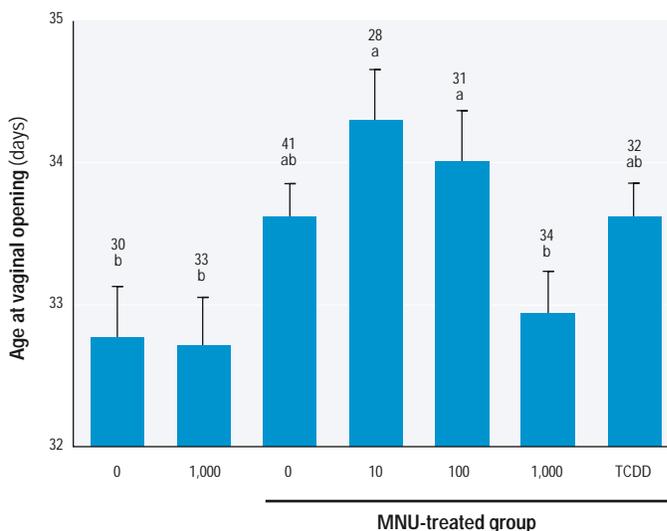


Figure 3. Effects of treatment on the age at vaginal opening occurrence. A significant ANOVA was obtained, and the Tukey-Kramer all-comparison test identified groups with significantly different means ($p < 0.05$). Bars with different letters are significantly different. The number above each bar represents the number of rats per treatment group.

Relevance to human disease. Given the absence of detectable effects at the lowest dose level, our study might suggest that in humans the neonatal level of exposure to the chemicals included in our mix does not modulate tumor development. Regardless of the changes in tumor phenotype in the MNU-100× dose group, the increase in the median number of lesions occurred at a dose representing 1,000 times the amount of PCBs (19 congeners), DDT, and DDE that a human baby would consume during the

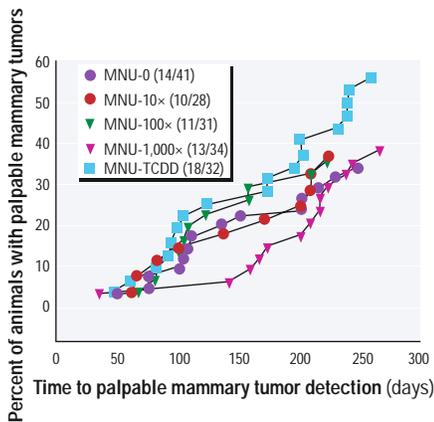


Figure 4. Mammary tumor incidence curves. Each point represents the cumulative percentage of rats that developed palpable tumors (benign and malignant) relative to the time of MNU injection (time = 0). The incidence curve of the MNU-TCDD group differs almost significantly ($p = 0.06$) from the MNU-1,000× group. None of the incidence curves significantly differed from the MNU-0 group. At the end of the experiment the percentage of rats that developed palpable mammary tumors was slightly increased ($p = 0.06$) by the MNU-TCDD (18/32 = 56% rats with palpable tumors) but not by the other treatments (MNU-1,000× 13/34, 38%; MNU-100× 11/31, 36%; MNU-10× 10/28, 36%) compared to controls (MNU-0 14/41, 34%).

first 24 days of life. In fact, the safety margin could be larger than 1,000. The concentrations of organochlorines in milk samples are not normally distributed, and the mixture contained non-ortho chlorinated PCBs based on mean levels, not the median levels; consequently, the proportions of non-ortho-chlorinated PCBs in the mixture exceed the proportions expected in most human milk samples. In addition, the amount of these PCBs in the mixture was slightly increased by the presence of eight times more PCB #77 than what we expected (Table 1). This difference might not have biologic consequences given that the TCDD-TEF for PCB #77 is 0.0001 (92), and it modifies the total TCDD-TEF exposure dose for the complete mixture from 31.42 to 31.44 ng. Nevertheless, a lack of information on the effects of more complete mixtures and on the effects occurring *in utero*, and a lack of understanding of the relations between toxicologic and carcinogenic processes, prevent us from ruling out the possibility of a link between TCDD, PCB, DDT, and DDE exposure and the risk of developing some types of breast cancer. Twenty-seven percent of the risk of developing breast cancer in twins derives from heritable factors (5). Twins are likely to receive similar exposures during the perinatal period, so the risk associated with *in utero* and/or neonatal exposure to organochlorines is likely to be smaller than 27%. Finally, the body burden in the human population is declining (69), which should decrease the risk for the general population even further.

The modulation of chemically induced mammary tumor development by organochlorines is an expensive but important experimental model for studying the initiation and the promotional phases of carcinogenesis. Moreover, it is one of the rare animal

models providing quantifiable late outcomes following *in utero* or neonatal exposure to low doses of contaminants. Given that in the absence of an initiator, organochlorines have no effects on mammary tumor development, more research is required using the MNU or DMBA model to understand their mechanisms of action and how they are modulated by environmental contaminants. Such studies should determine the usefulness of this model as an indicator of adverse effects in humans, and could be used to identify epidemiologic markers of breast cancer susceptibility, and thus improve epidemiologic investigations.

In summary, we have shown that exposure during the neonatal period to TCDD or a mixture of PCBs, DDT, and DDE, before MNU treatment at neonatal day 21 increased the median number of mammary lesions (palpable and microscopic, benign and malignant) developing in the adult rat. The percentage of rats that developed palpable tumors (benign plus malignant) was also higher in the MNU-TCDD group than in the control group. The highest dose of the mixture delayed the development of palpable tumors, but the MNU-TCDD treatment did not. Perhaps treatments with TCDD or the highest dose of the mixture sensitize the mammary tissue to the carcinogenic effects of MNU by altering the initiation/early promotion phase of the carcinogenic processes. Then, the MNU-1,000× group exerted a transient inhibition of promotional mechanisms up to approximately 150 days after the MNU injection, or selected tumorigenic tissues responsive only to the late promotional factors. We are now assessing the dose-response effects of neonatal exposure to Ah-R agonists in human milk on the development of mammary lesions. It will remain

Table 4. Incidence (percent of animals) of nonmammary tumors and organ abnormalities from tissues dissected during necropsy.

Nonmammary tumors/ abnormalities	0 (n = 30)	1,000× (n = 33)	MNU-treated mixture				
			0 (n = 41)	10× (n = 28)	100× (n = 31)	1,000× (n = 34)	TCDD (n = 32)
Fibroma	0	0	2.4	0	0	0	0
Ameloblastoma ^a	0	0	2.4	0	0	0	0
Keratoacanthoma ^b	0	0	2.4	3.6	0	0	3.1
Fibrohistiocytic tumor	0	0	0	0	0	0	3.1
Epidermoid cyst	0	0	2.4	0	0	8.8 ^c	0
Abscessed salivary gland	0	0	0	0	0	0	3.1
Kidney ^d	3.3	0	7.3	10.7	3.2	2.9	6.3
Ovary ^e	0	0	14.6	17.9	6.5	17.6	12.5
Uterus	3.3 ^f	0	4.9 ^g	3.6 ^h	0	2.9 ⁱ	0
Adrenal/ ^j	0	0	2.4	7.1 ^k	3.2	2.9	0
Pituitary gland ^l	0	0	4.9	0	3.2	2.9	3.1
Spleen	0	3.0 ^m	0	0	0	0	3.1 ⁿ
Fur loss	0	0	0	3.6	3.2	0	3.1

^aTooth-related. ^bSkin tumors. ^cOne animal had three epidermoid cysts. ^dAll abnormal kidneys (12 in total) were renal mesenchymal tumors, except for one (MNU-0) which was glomerulonephritis. In one animal (MNU-10×) both kidneys were abnormal. ^eAll ovarian follicular cysts (abnormally large clear follicles), except one thecoma (MNU-TCDD), one hemorrhagic ovarian cyst (MNU-10×), and one atrophic ovary (MNU-10×). ^fNot histologically classified, uterine bleeding. ^gOne enlarged uterus (not classified), one with an endometrial polyp. ^hEndometritis. ⁱFocal cystic hyperplasia. ^jAdrenals were abnormally enlarged, but not histologically classified except for one (MNU-1,000×) which was a hemorrhagic infarction (not specific to treatment). ^kOne enlarged adrenal was next to a kidney with a renal mesenchymal tumor. ^lAbnormal pituitary glands were not histologically classified. A dark area was observed on three pituitaries (MNU-0, MNU-100×, and MNU-TCDD groups), a white spot on an MNU-0-treated, and an MNU-1,000×-treated pituitary gland had blood inside. ^mEnlarged, not classified. ⁿTwo small cysts on side of spleen, not classified.

to be determined whether differences in the development of mammary lesions between treatment groups resulted from immediate toxic effects at the time of MNU injection, from persistent toxic effects, or from long-term effects altering the physiology of aging.

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